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Estren Is a Selective Estrogen Receptor Modulator with Transcriptional Activity

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ABSTRACT

It was recently reported that the synthetic compound estren increases bone mass without affecting reproductive organs or classic transcription. The aim of the present study was to further characterize the in vivo and in vitro effects of estren. We demonstrate that estren is a selective estrogen receptor modulator (SERM) with a strong effect on thymus, a moderate effect on uterus and trabecular bone, but no major effect on fat or cortical bone in 11-month-old ovariectomized mice. The effect of estren on trabecular bone and uterus is mediated via estrogen receptors (ERs) because no effect is seen in ER double-inactivated mice. Furthermore, with the use of ERα- and ERβ-expressing reporter cell lines, we demonstrate that estren displays an agonistic effect on transcriptional activity of an estrogen-responsive element-driven reporter gene with a degree of agonism similar to that of 17β-estradiol for both ERα and ERβ. Thus, estren has the capacity to exert genomic effects via both ERα and ERβ. We conclude, in contrast to what was previously reported by others, that estren is a SERM with transcriptional activity.

Estrogens and androgens are of importance for the regulation of bone metabolism in both males and females. Skeletal effects of sex steroids are mediated via estrogen receptors (ERs) and the androgen receptor (AR), which are nuclear receptors with transcriptional activity. However, a variety of cell types respond rapidly to estrogens within seconds or minutes, making a classic genomic mechanism of action unlikely (Wehling, 1997; Brubaker and Gay, 1999; McEwen and Alves, 1999; Compston, 2001), and there are now several pieces of evidence supporting the notion of nongenomic functions of sex-steroid receptors (Endoh et al., 1997; Brubaker and Gay, 1999; Migliaccio et al., 2000; Simoncini et al., 2000; Compston, 2001; Duan et al., 2001; Kousteni et al., 2001). Furthermore, Kousteni et al. (2001) proposed that ERα, ERβ, or AR could transmit nongenomic antiapoptotic effects on osteoblasts in vitro with similar efficiency, irrespective of whether the ligand is an estrogen or an androgen. It has been suggested that the genomic mechanisms mediate the reproductive effects, whereas the nongenomic effects are responsible for the bone-sparing effect of estrogens (Kousteni et al., 2001, 2002). The synthetic compound 4-estren-3α,17β-diol (estren) has recently been described to increase bone mass without affecting reproductive organs or classic transcription (Kousteni et al., 2001, 2002). The statement of an absence of transcriptional activity for estren is derived from an in vitro experiment in which estren did not induce C3 transcription in ERα-transfected HeLa cells (Kousteni et al., 2001, 2002). The aims of the present study were the following: 1) to determine the tissue specificity for the effect of estren; 2) to determine whether the in vivo effect of estren is mediated via ERs and/or AR with the same efficiency; and 3) to determine in vitro, using ER-expressing reporter cell lines, whether estren has any transcriptional activity. We demonstrate here that estren is a selective estrogen receptor modulator (SERM) with transcriptional activity.

Materials and Methods

Animals. Male and female double-heterozygous (ER$\alpha^{+/-}\beta^{+/-}$) mice were mated, resulting in WT, ER$\alpha^{-/-}$ER$\beta^{+/+}$ (ER$\alpha^{-/-}$), ER$\alpha^{+/+}$ER$\beta^{-/-}$ (ER$\beta^{-/-}$) and ER$\alpha^{-/-}$ER$\beta^{-/-}$ (ER$\alpha^{-/-}\beta^{-/-}$) offspring with a mixed C57BL/6J/129 background (Lubahn et al., 1993; Krege et al.,

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ABBREVIATIONS: ER, estrogen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; SERM, selective estrogen receptor modulator; AR, androgen receptor; BMD, bone mineral density; BMC, bone mineral content; ovx, ovariectomized; WT, wild type; pQCT, peripheral quantitative computerized tomography; FCS, fetal calf serum; ALP, alkaline phosphatase; 293/hERα, human 293 kidney cells expressing estrogen receptor α; 293/hERβ, human 293 kidney cells expressing estrogen receptor β; ICI 182,780, fulvestrant.

1998). Genotyping of tail DNA was performed at 3 weeks of age as described previously (Vidal et al., 2000). Animals had free access to fresh water and soy-free food pellets (R70; Lactamin AB, Stockholm, Sweden). At 11 months of age, mice were ovariectomized (ovx) and injected daily subcutaneously with 17β-estradiol benzoate (0.7 μg/mouse) (Sigma Chemical, St. Louis, MO) or estren (75 μg/mouse) (Steraloids, Newport, RI) for 4 weeks. Control mice received injections of vehicle oil (olive oil; Apoteksbolaget, Göteborg, Sweden).

Peripheral Quantitative Computerized Tomography. Computerized tomography was performed with the Stratec peripheral quantitative computerized tomography (pQCT) XCT Research M (version 5.4B; Norland Corporation, Fort Atkinson, WI) as described previously (Windahl et al., 1999). Trabecular bone mineral density (BMD) was determined ex vivo, with a metaphyseal pQCT scan of the proximal tibia and defined as the inner 45% of the total cross-sectional area. Cortical bone parameters were determined ex vivo with a mid-diaphyseal pQCT scan of the femur.

Generation of Stable ERα and ERβ Reporter Cell Lines. Generation of stable human embryonic kidney 293 cells (American Type Culture Collection no. CRL 1573) expressing human ERα and human ERβ and the pΔERE2-ALP reporter vector has been described previously (Barkhem et al., 1998). All cell lines were cultured routinely at 37°C in humidified chambers at 5% CO_2 in minimal essential medium (phenol red-free) supplemented with 10% FCS and 2 mM L-glutamine.

Assay Procedure for Hormonal Effects on 293/hERα and 293/hERβ Reporter Cells. Cells (25×10^3 per well) were seeded onto 96-well culture plates in 100 μl of Coon's/F12 (phenol red-free) supplemented with 10% FCS (stripped twice using dextran-coated charcoal) and 2 mM L-glutamine. Twenty-four hours later, conditioned medium was replaced with 100 μl Coon's/F12 supplemented with 1% FCS (stripped twice using dextran-coated charcoal), 2 mM L-glutamine, gentamicin (50 μg/ml), and hormonal substances as indicated in the figure legends. In all experiments, cells were exposed to hormones for 72 h before harvest and analysis for effect on reporter-gene expression. Triplicate determinations of reporter protein levels in the conditioned media for each concentration of compound were performed in all experiments.

Assay for Human Placental Alkaline Phosphatase. The level of alkaline phosphatase (ALP) expressed from the ΔERE2-ALP reporter vector in the stably transformed 293/hERα and 293/hERβ reporter cells was determined using a chemiluminescent assay as follows: a 10-μl aliquot of heat-treated (at 65°C for 30 min) conditioned cell-culture medium was mixed with 200 μl of assay buffer (10 mM diethanolamine, pH 10.0, 1 mM $MgCl_2$, and 0.5 mM CSPD) in white microtiter plates (Dynatech Labs, Chantilly, VA) and incubated at 37°C for 20 min before being transferred to a microplate-format luminescence counter (1450 Microbeta; PerkinElmer Wallac, Turku, Finland). The setting of the Microbeta was for a 1-s reading of each well. The ALP activity is expressed in luminescence counts per second, which is directly proportional to the level of ALP expressed from the cells.

Results

Tissue Specificity for the Effect of Estren Compared with the Effect of 17β-Estradiol. To determine the tissue specificity for the effect of estren compared with the effect of 17β-estradiol, ovx mice were treated with vehicle, estren, or 17β-estradiol (0.7 μg/mouse/day). Surprisingly, already at an estren dose of 75 μg/mouse/day, a clear effect was seen on the uterine weight (132 ± 28% over vehicle), which is in contrast to a previous study using a 70% higher dose of estren and demonstrating no uterine effect (Kousteni et al., 2002). The effects of estren on different estrogen-responsive tissues were then compared with the effects of a physiological dose of 17β-estradiol (Lindberg et al., 2002). Both estren and 17β-estradiol increased the uterine weight compared with vehicle in ovx mice (Fig. 1). The effect of estren on the uterine weight was 16% of the effect exerted by 17β-estradiol (Table 1). As expected, the weights of the gonadal and the retroperitoneal fat deposits were reduced by 17β-estradiol in ovx mice. In contrast, no effect on these fat deposits was seen by estren treatment (Fig. 2). It is well known that estrogen treatment decreases the weight of thymus (Marotti et al., 1984). Both 17β-estradiol and estren treatment decreased the thymus weight, and for this parameter, the effects of estren and 17β-estradiol were of the same magnitude (17β-estradiol, −59 ± 11%; estren, −42 ± 6% compared with vehicle) (Table 1). Both estren and 17β-estradiol increased the trabecular BMD, and the effect of estren on trabecular BMD was 22% of the effect exerted by 17β-estradiol (Fig. 3A and Table 1). In contrast, cortical bone parameters, including cortical bone mineral content (BMC), cortical cross-sectional area, and cortical thickness, were increased by 17β-estradiol but not by estren (Fig. 3B, Table 1, and data not shown).

The Effects of Estren Are Mediated Via Estrogen Receptors. Because previous in vitro data have indicated that the effect of estren is mediated via ERs or the AR with similar efficiency (Kousteni et al., 2001, 2002), we designed an experiment to determine whether the in vivo effects of estren can be mediated via both the ERs and the AR. The in vivo effects of estren and 17β-estradiol were investigated in WT and $ER\alpha^{-/-}\beta^{-/-}$ mice. The regulatory effects of estren and 17β-estradiol on uterus, fat, trabecular bone, and cortical bone, observed in WT mice, were lost in $ER\alpha^{-/-}\beta^{-/-}$ mice (Figs. 1–3). Thus, the in vivo effects of estren and 17β-estradiol on uterus weight and trabecular bone are mediated via ERs, and the AR cannot replace the ERs for the mediation of these effects (Figs. 1–3).

Estren has Transcriptional Activity. The in vivo experiments demonstrated that the effects of estren are mediated via ERs. To determine whether estren has any transcriptional activity mediated via ERs, the effect of estren on the activation of an estrogen-responsive element-driven reporter gene (ALP) was tested in vitro in human 293 kidney epithe-

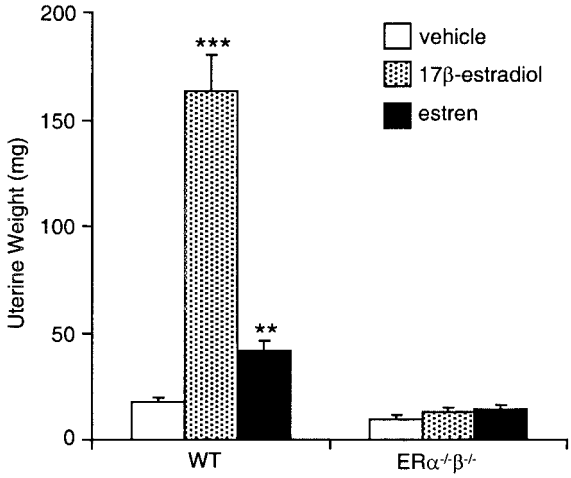

Fig. 1. Weight of uterus. Eleven-month-old female WT and $ER\alpha^{-/-}\beta^{-/-}$ mice were ovariectomized and then treated with vehicle, 17β-estradiol (0.7 μg/mouse/day), or estren (75 μg/mouse/day) for 4 weeks (n = 7–8). Values given are means ± S.E.M. ⋆⋆, $p < 0.01$, ⋆⋆⋆, $p < 0.001$ versus vehicle, Student's t test followed by post hoc analysis according to Bonferroni test.

lial ERα (293/hERα)- and ERβ (293/hERβ)-expressing reporter cell lines (Barkhem et al., 1998). The responses of 293/hERα and 293/hERβ to estren are shown in Fig. 4, A and B, and expressed as the percentage of agonism of 17β-estradiol. Estren displayed a full agonistic effect on transcriptional activity with a degree of agonism similar to that of 17β-estradiol for both receptors (99% agonistic activity via ERα and 87% agonistic activity via ERβ compared with the activity of 17β-estradiol) (Fig. 4, A and B). As expected from previous ERα and ERβ binding studies (Kousteni et al., 2002), the transcriptional potency of estren was lower than that of 17β-estradiol in both the 293/hERα and the 293/hERβ cell lines (Fig. 4, A and B). The ER antagonist ICI 182,780 antagonized, in a dose-dependent manner, the effect of estren in both the 293/hERα and the 293/hERβ cells. The potency of ICI 182,780 in antagonizing estren-induced gene expression was similar in both reporter cell lines (IC_{50} values: 0.75 nM for ERα and 0.57 nM for ERβ) (Fig. 4, C and D). Thus, estren is a full agonist for the transcriptional activity mediated via both ERα and ERβ.

Discussion

Estrogen replacement therapy is beneficial for the treatment of postmenopausal bone loss but is reported to have undesirable effects on reproductive tissue and is associated with an increased risk of breast cancer (Riggs and Hartmann, 2003). SERMs having the beneficial effects of estrogen in bone with reduced side effects in breast and uterus have been developed. However, currently available SERMs are less potent in bone than estrogen replacement therapy (Delmas et al., 1997). Therefore, the recent description of estren as a potent stimulator of bone mass and strength without any side effects on reproductive organs has been very much appreciated (Kousteni et al., 2001, 2002). Furthermore, the described mode of action for the bone-sparing effect of estren is unique because it was reported to be a nongenomic effect involving functional and direct interactions with components of the Src/Shc/extracellular signal-regulated kinase signaling pathways. Extranuclear signaling by classic ERs and AR is supported by numerous reports describing interactions between sex-steroid receptors and components of the intracellular signaling machinery including phosphatidylinositol-3-phosphate kinase, Src, and Shc (Migliaccio et al., 2000; Simoncini et al., 2000; Duan et al., 2001; Kousteni et al., 2001). The recently described extranuclear mode of action of estren in bone has led to the definition of a novel class of mechanism-specific substances called ANGELS (Activator of NonGenotropic Estrogen-Like Signaling) (Manolagas et al., 2002). However, the present study indicates that estren rather, in a wider perspective, is a SERM with transcriptional activity.

The results of our present characterization of the effects of estren are in conflict with two previous publications from one research group describing the in vitro and in vivo effects of estren (Kousteni et al., 2001, 2002). In the in vivo study by Kousteni et al., it was found that estren had no effect on the uterine weight, whereas in the present study, using even a slightly lower dose of estren than in the previous study, a clear estren-induced increase in uterine weight was seen. In agreement with the previous study, the amount of trabecular bone was increased by estren in the present study. However,

TABLE 1

Tissue specificity for the effect of estren compared with the effect of 17β-estradiol on uterine weight, gonadal fat weight, thymus weight, trabecular BMD, and cortical BMC

Eleven-month-old female mice were ovariectomized and then treated for 4 weeks with vehicle, 17β-estradiol, or estren ($n = 8$). Values for the effect of estren compared with the effect of 17β-estradiol are given as the effect of estren divided by the effect of 17β-estradiol and are expressed as a percentage (equal to the percentage of estrogenic activity of estren) and as the relative tissue specificity for the estrogenic activity of estren when the effect on uterus is given as 1 (equal to the relative tissue specificity of estren).

Parameter	Estrogenic Activity of Estren	Relative Tissue Specificity of Estren (Uterus = 1)
	%	
Uterine weight	16.2	1
Gonadal fat weight	0	0
Thymus weight	71	4.4
Trabecular BMD	21.8	1.3
Cortical BMC	0	0

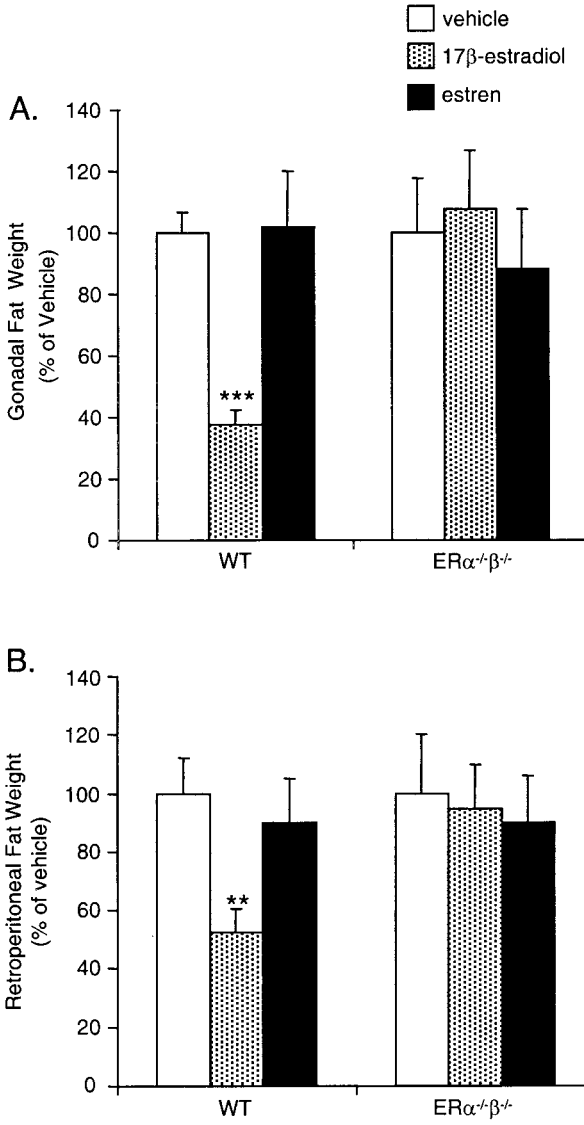

Fig. 2. Weight of gonadal (A) and retroperitoneal (B) fat deposits. Eleven-month-old female WT and $ER\alpha^{-/-}\beta^{-/-}$ mice were ovariectomized and then treated with vehicle, 17β-estradiol (0.7 μg/mouse/day), or estren (75 μg/mouse/day) for 4 weeks ($n = 8$). Values are given as means ± S.E.M. **, $p < 0.01$, ***, $p < 0.001$ versus vehicle, Student's t test followed by post hoc analysis according to Bonferroni test.

in our study, the estrogen-like activity of estren was of the same magnitude on uterus as on trabecular bone, whereas in the previous study, a clear effect was seen on bone without any effect on uterus. It is difficult to explain the different results of the two studies. One may speculate that the 60-day slow-release treatment in the previous study was active during most of the time but not during the last few days of treatment. This would then result in bone, a slow-responding tissue, being preserved, with the uterus, a fast-responding tissue, no longer showing any effects of estren at the end of the treatment period. In contrast, in the present study, estren was given as subcutaneous daily injections during the whole experiment to ensure that the animals received the treatment until the final analysis.

In the present study, the effect of estren on several different tissues was investigated and compared with the effect of 17β-estradiol. It was demonstrated that estren exerts a relatively strong effect on thymus weight, a medium effect on uterus weight and trabecular bone, and no effect on fat mass or cortical bone. Thus, the degree of the estrogen-like activity of estren is tissue-specific.

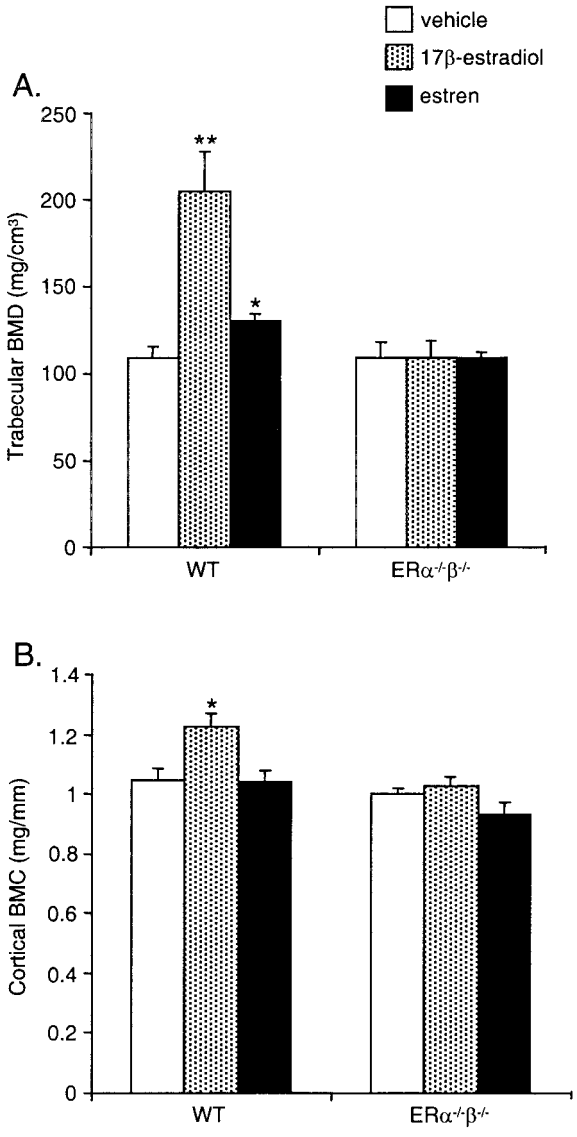

Fig. 3. A, trabecular volumetric BMD of the metaphyseal area of the proximal tibia measured using pQCT. B, cortical BMC as determined using a mid-diaphyseal pQCT scan of the femur. Eleven-month-old female WT and ER$\alpha^{-/-}\beta^{-/-}$ mice were ovariectomized and then treated with vehicle, 17β-estradiol (0.7 μg/mouse/day), or estren (75 μg/mouse/day) for 4 weeks ($n = 8$). Values are given as means ± S.E.M. ⋆⋆, $p < 0.01$, ⋆$p < 0.05$ versus vehicle, Student's t test followed by post hoc analysis according to Bonferroni test.

Previous in vitro data have indicated that the effect of estren might be mediated via ERs or the AR with similar efficiency (Kousteni et al., 2001, 2002). Here, we demonstrate in vivo that the effects of estren on trabecular bone and uterus are mediated via ERs and that the AR cannot replace the ERs in mediating these effects. We also demonstrate that the trabecular bone-sparing effect of 17β-estradiol is mediated only via ERs and not via the AR. Furthermore, the bone-sparing effect of 5α-dihydrotestosterone-induced AR stimulation is not dependent on the ERs (Movérare et al., 2003). Therefore, in contrast to what has recently been concluded from in vitro studies (Kousteni et al., 2001), there is no cross-reactivity between ERs and AR for the mediation of the trabecular bone-sparing effect of sex steroids, and the effect of estren in vivo is only mediated via ERs. Unfortunately, because of a complex breeding procedure of the ER-inactivated mice, no sham-operated control group was included in the present study. However, we have in a previous study seen that the 17β-estradiol dose given to the ovx mice in the present study results in sham-operated control levels for the different estrogen-responsive parameters analyzed in the present study (data not shown).

Because of an in vitro finding that estren did not induce C3 transcription in ERα-transfected HeLa cells, it was postulated that estren has no transcriptional activity and that all of its effects must be nongenomic (Kousteni et al., 2001). In contrast, using another well-established in vitro system of human 293 kidney epithelial ERα- and ERβ-expressing reporter cell lines (Barkhem et al., 1998), we demonstrated that estren displays a full agonistic effect on transcriptional activity with a degree of agonism similar to that of 17β-estradiol for both ERα and ERβ. The specificity of this effect was confirmed by the result that the ER antagonist ICI 182,780 antagonized, in a dose-dependent manner, the effect of estren on the transcriptional activity mediated via both receptors. Thus, estren has the capacity to exert genomic effects via both ERα and ERβ. In addition, we have also monitored the genomic response to estren in the human endometrial carcinoma cell line Ishikawa (Littlefield et al., 1990) by analyzing the expression of the endogenous alkaline phosphatase gene. Similar to the response in the genetically engineered 293 ERα and ERβ reporter cell lines, estren generated an agonist response that was totally blunted by the pure ER antagonist ICI 182,780 (data not shown), supporting the notion that estren exerts ER-mediated genomic effects.

The conflicting results in the previous study using HeLa cells compared with the present study using 293 kidney epithelial cells might depend on the choice of promoter and/or the choice of cell line. It is well known that the effect of ERs is exclusively mediated via AF-2 in HeLa cells (Berry et al., 1990; Metivier et al., 2000), whereas in the 293 kidney epithelial cells, the activity of the ERs is dependent on the function of both AF-1 and AF-2. The lack of transcriptional activity of estren in HeLa cells might indicate that the agonist effect of estren on ERs requires functionality of both AF-1 and AF-2 (Berry et al., 1990; Metivier et al., 2000). We do not doubt that estren, in analogy to 17β-estradiol, also

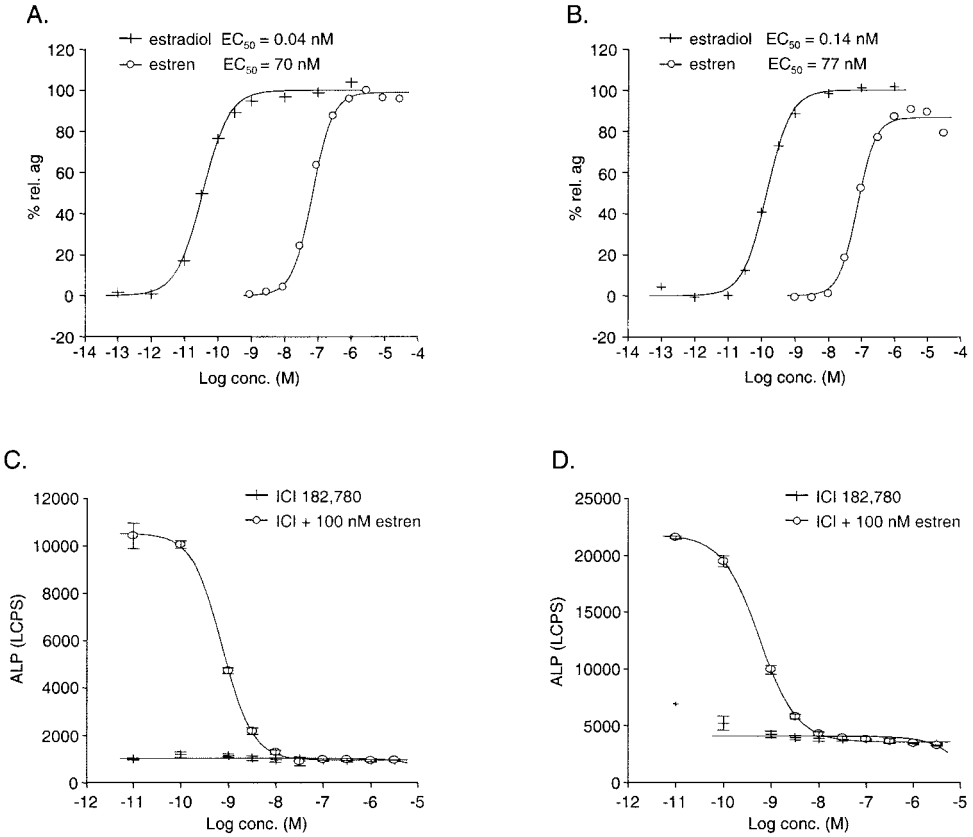

Fig. 4. Effect of estren and 17β-estradiol on transcriptional activity. Response to increasing concentrations of 17β-estradiol and estren in the 293/hERα (A) and 293/hERβ (B) reporter cell lines. The level of ALP reporter protein expressed was analyzed 72 h after the addition of the ligands. Antagonist response to increasing concentration of ICI 182,780 in the presence or absence of estren (100 nM) in 293/hERα (C) and 293/hERβ (D) reporter cell lines. The response values for each concentration of ligand are the means of triplicate determinations with the means ± S.D. for each value indicated.

elicits nongenomic responses, but our data clearly indicate, in contrast to the data from the study by Kousteni et al. (2002), that estren also has typical genomic effects. Thus, our data suggest that the biological effect of estren cannot solely be explained by a nongenomic mechanism but that genomic mechanisms of estren also have to be considered.

In conclusion, our results demonstrate that estren is a SERM with effects on uterus, trabecular bone, and thymus but without major effect on fat or cortical bone. The effects of estren on bone and uterus are mediated via ERs, and the AR cannot replace the ERs for these effects. Furthermore, it is clear that estren has the capacity to exert genomic effects mediated via ERs.

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